A New Synthetic Protease Inhibitor, E-3123, Reduces Organelle Fragility of Acinar Cells in Rat Caerulein Pancreatitis

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Arch Jpn Chir 60(6), 406～414, Nov., 1991

A New Synthetic Protease Inhibitor, E-3123, Reduces Organelle Fragility of Acinar Cells in Rat Caerulein Pancreatitis

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Received for Publication, August 30, 1991.

Abstract

The present study investigated the protective effect of a new potent synthetic protease inhibitor, E-3123 (4-guanidinobenzoate methanesulfonate) on the exocrine pancreas in the caerulein induced experimental pancreatitis both in-vivo and in-vitro at 3 different doses (1, 2, and 5 mg/kg・hr).

This protease inhibitor prevented hyperamylasemia, pancreatic edema, congestion of amylase, and both amylase and lactic dehydrogenase (LDH) discharge from dispersed acini, as well as cathepsin B leakage from lysosomes and malate dehydrogenase (MDH) leakage from mitochondria in a dose-dependent manner, particularly in doses of 2 and 5 mg/kg・hr. Furthermore, the combined prophylactic and therapeutic use of this agent seems to be very effective in preventing caerulein induced pancreatitis.

These results indicate that E-3123 plays its protective roles against pancreatitis in the subcellular compartment: lysosomes, mitochondria, cellular or organella membranes. It is hoped that such a low molecular weight protease inhibitor as E-3123 will be clinically useful in the treatment of acute pancreatitis.

Introduction

It has been reported that edematous pancreatitis characterized by hyperamylasemia, can be induced by infusing rats with a dose of caerulein in excess of that which causes maximal rates of digestive enzyme secretion from the pancreas[10,15,17,21]

In this caerulein-induced pancreatitis, the lysosomal functions are impaired and lysosomal fragility increased in acinar cells[18,26]. The colocalization of lysosomal and digestive enzymes in acinar cells are thought to play a triggering role in the pathogenesis of acute pancreatitis, because the lysosomal enzymes can activate digestive enzymes[5,16,18,22,26]. Thus in the early stage of pancreatitis lysosomal enzyme as well as trypsin, seems to play a key role in the development of the disease.

In this communication we report the results of studies on the effects of new potent low molecular weight protease inhibitor, E-3123, 4-(2-succinimidoethylthio) phenyl 4-guanidinobenzoate

Key words: Caerulein pancreatitis, E-3123, Cathepsin, Malate dehydrogenase.
索引語：セルレイン膵炎，E-3123，カテプシン，マレートデハイドロゲナーゼ.
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methanesulfonate[8] in this carulein-induced pancreatitis. E-3123 has been found to inhibit many enzymes, such as trypsin, plasmin, thrombin, kallikrein, phosphalipase A_2 and elastase[8] In accord with other previous reported synthetic protease inhibitors[12,23,24,27], it is hoped that E-3123 will also protect the pancreatic acinar cells at subcellular levels in the pathogenesis of acute pancreatitis.

Materials and Methods

Male Wistar rats (225–250 g) (Shizuoka Experimental Animals, Shizuoka, Japan) were used for this experiment. All the animals were given free access to tap water and commercial diet in temperature (23±3°C) regulated and light-dark cycle (light, 5:00–17:00) regulated animal quarters. After a 16-hour fast, a V-3 cannula (Insul-Tab, Inc., Woburn, MA, U.S.A.) was placed via the right external jugular vein into the superior vena cava under light intraperitoneal sodium pentobarbital anesthesia (15 mg/kg), and its patency was maintained by continuous infusion of heparinized (30 U/ml) 150 mM NaCl solution at a speed of 0.21 ml/hr, with an infusion pump. The animals were housed in shoe-box cages, allowed 12 hours to recover from the effects of anesthesia and surgery, and given free access to tap water and diet before the next caerulein infusion.

Animals were subsequently divided into the following 4 groups: (a) 15 control rats (CONT)—infused only with heparinized saline at a speed of 0.58 ml/hr for 3.5 hours. (b) 18 caerulein rats (CER)—infused with heparinized saline as noted above (0.58 ml/hr for 3.5 hours), but with caerulein (Sigma Chemical, St. Louis, MO, U.S.A.) added to the infusate so that each animal received 5 μg/kg · hr. (c) 54 E-3123 plus caerulein rats (E_1, E_2, E_3)—identical to the caerulein group, but E-3123 was infused for 2 hours before and throughout the 3.5 hours of caerulein infusion in 3 different doses, 1, 2 and 5 mg/kg · hr. (d) 18 E-3123 plus caerulein rats (E_4)—identical to caerulein group, but E-3123 was infused only throughtout the 3.5 hours of caerulein infusion in a dose of 5 mg/kg · hr.

During these infusions, tap water and diet were removed from the cages. E-3123 was kindly donated by Eisai Pharmaceuticals, Co., Ltd, Tokyo, Japan.

Serum amylase levels, pancreatic water content, pancreatic amylase and cathepsin B content

At selected times (after 3.5 hours of infusions) rats were killed with a large dose of intravenous pentobarbital after blood had been drawn from the IVC for the determination of serum amylase levels. Portions of the pancreas were removed quickly and trimmed of fat. One small portion of the pancreas was used for the quantitation of pancreatic edema by a comparison of the weight immediately after removal (wet weight) with that of the same sample after incubation at 150°C for 48 hours in a dessicator (Isotemp, Fisher Scientific, Fair Lawn, NJ, U.S.A.) (dry weight).

Other small portions of the pancreas were homogenized in 5 ml of cold phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100 (Fisher Scientific) with a Brinkmann Polytron (Brinkmann Instruments, Inc, Westbury, NY, U.S.A.) for the measurement of pancreatic amylase and cathepsin B content. Both amylase and cathepsin B activity, as well as deoxyribonucleic acid (DNA) concentration, were measured in the resulting supernatant after low speed centrifugation (150 × g at 4°C for 15 min), and both were expressed as U/mg DNA.

Amylase and lactic dehydrogenase (LDH) discharge from dispersed pancreatic acini

From the another rat in each group, dispersed pancreatic acini were prepared by collagenase (Cooper Diagnostics, Freehold, NJ, U.S.A.) digestion and gentle shearing, as previously described[14] Acini were suspended in HEPES-Ringer buffer (pH 7.4) containing NaCl (115 mM),

KCl (5 mM), $MgSO_4$ (1 mM), Na_2HPO_4 (1 mM), $CaCl_2$ (1.26 mM), glucose (15 mM), and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (Sigma Chemical) (10 mM). In addition, the buffer contained Eagles' basal amino acids, bovine serum albumin (0.1%) (Sigma Chemial) and soybean trypsin inhibitor (0.01%) (Cooper Diagnostics) and was preoxygenated by 100% O_2 bubbled through it. The acini were incubated in this buffer in an atmosphere of O_2 in a shaking water bath maintained at 37°C. At 30-minute intervals (up to 120 min) aliquots were removed, and LDH activity in both the suspending medium and the pelleted acini was measured. LDH discharge from acini was expressed as a percent of total LDH activity present in the acini at the onset of in-vitro incubation. In the same samples amylase activity was measured and amylase discharge from acini was expressed in the same way as LDH discharge.

Cathepsin B leakage from lysosomes and malate dehydrogenase (MDH) leakage from mitochondria

At selected times other rats from each group were sacrificed, and portions of the pancreas were removed, trimmed of fat, and homogenized in ice-chilled 5 mM MOPS buffer as described above. This homogenate was centrifuged (150×g at 4°C for 15 min) to removed unbroken cells and debris. The resulting supernatant was centrifuged (12,000×g at 4°C for 12 min) to obtain a combined zymogen-lysosome-mitochondria-rich pellet. This pellet, arbitrarily considered to contain 100% of both lysosomal and mitochondrial enzyme activity, was resuspended in 5 mM MOPS buffer and incubated for various intervals (30, 60, 90, and 120 min) at 25°C in a shaking water bath. The samples were then recentrifuged (12,000×g at 4°C for 12min) to separate the particulate from the soluble lysosomal activity, each of which was individually measureed after separation of the pellet and supernatant. As a lysosomal enzyme, cathepsin B activity was measured in both the pelleted and the soluble fractions. Centrifugation and subsequent measurement of particulate and soluble lysosomal enzyme activity identified the rate and extent of in-vitro rupture of lysosomal enzyme-containing organelles. Soluble cathepsin B activity was expressed as a percent of the total cathepsin B activity as an index of lysosomal fragility. In the same samples, malate dehydrogenase (MDH) activity as a mitochondrial enzyme was measured, and the MDH leakage from mitochondria was expressed in the same way as cathepsin B leakage.

Assays

Amylase activity was measured with soluble starch (Sigma Chemical) as the substrate by the method of Bernfeld[3]. One unit (U) of amylase activity was defined as that which released 1 mg of maltose from the substrate per minute at 30°C. Cathepsin B activity was measured fluorometrically with CBZ-arginyl-arginine-β-naphthylamide (Bachem Bioscience, Inc., Philadelphia, PA, U.S.A.) as the substrate, as described by McDonald and Ellis[13]. One unit (U) of cathepsin B activity was defined as that which released 1 nano mole of β-naphthylamine (Sigma Chemical) from the substrate per minute (at 37°C). Deoxyribonucleic acid (DNA) concentration was measured by the method of LaBarca and Paigen[9] with calf thymus DNA (Sigma Chemical) as the standard. Lactic dehydrogenase (LDH) activity and malate dehydrogenase (MDH) activity were measured by the mthod of Bergmeyer and co-workers[1,2], for determination of the rate consumption of pyruvate and of reduced diphosphopyridine nucleotide (β-NADH) (Sigma Chemical), and that of oxaloacetic acid and reduced diphosphopyridine nucleotide, respectively.

Analysis of data

The results reported in this communication represent the mean±SEM for n determinations. Differences between groups were evaluated by ANOVA with the Tukey method and significant di-

fferences are those associated with a probability value (p) of less than 0.05.

Results

Serum amylase levels, pancreatic water content, pancreatic amylase and cathepsin B content

In agreement with previous report, infusion of a supramaximal dose of caerulein (5 μg/kg · hr) for 3.5 hours was found to cause an increase in serum amylase levels and pancreatic edema. This latter could be objectively quantitated by the pancreatic water content. The infusion of E-3123 (1, 2 and 5 mg/kg · hr) before and during the caerulein infusion markedly reduced the serum amylase levels. These effects were dose-related and significant. The administration of 5 mg/kg · hr of E-3123 only during caerulein infusion also had a significant effect, but it was less effective than when given both prophylacticly and therapeuticly. In addition, pancreatic edema expressed as pancreatic water content was also greatly reduced by the prophylactic and therapeutic use of E-3123 in combination. These effects were also dose-dependent and significant. The administration of 5 mg/kg · hr of E-3123 only during caerulein infusion also had a significant effect, but it was less effective than the combined prophylactic and therapeutic treatment.

The administration of E-3123 in doses of 2 and 5 mg/kg · hr reduced the elevated amylase con-

Table 1 Effect of E-3123 on serum amylase and pancreatic water, amylase and cathepsin B content in rat caerulein-induced acute pancreatitis.

Group	n	Serum amylase (U/ml)	Pancreatic water content (%)	Pancreatic amylase content (U/mg DNA)	Pancreatic cathepsin B content (U/mg DNA)
CER	6	25±2	86±2	685±41	2443±521
E_1	6	14±2*	80±2*	604±39	2248±312
E_2	6	10±2**	76±3**	542±27*	1976±293
E_3	6	7±1**	74±1***	488±31***	1902±175
E_4	6	12±2*	78±2*	553±25*	2016±211
CONT	5	5±1***	73±2***	478±32***	1876±273

The values are expressed as mean±SEM

CER, caerulein group; E_1–E_4, caerulein plus E-3123 group; CONT, control group; *, $p<0.05$; **, $p<0.02$; and ***, $p<0.01$, compared with CER group.

Table 2 Effect of E-3123 on LDH discharge from dispersed acini during acute pancreatitis induced by supramaximal dose of caerulein.

Group	n	LDH discharge from dispersed acini (% of total) Incubation time (min)			
		30	60	90	120
CER	6	7±1	10±1	15±2	19±2
E_1	6	6±1	8±1	11±2	15±2
E_2	6	4±1	7±1*	10±1*	13±2*
E_3	6	4±1	6±1*	9±1*	12±2*
E_4	6	5±1	7±1*	10±2*	13±1*
CONT	5	4±1	6±1*	9±2**	11±2*

The values are expressed as mean±SEM and the percent of total activity.

CER, caerulein group; E_1–E_3, caerulein plus E-3123 in doses of 1, 2 and 5 mg/kg·hr; E_4, caerulein plus 5 mg/kg·hr of E-3123 only during caerulein infusion; *, $p<0.05$; **, $p<0.02$, compared with CER group.

tent significantly and dose-dependently. The administration of E-3123 in a dose of 5 mg/kg・hr only during the caerulein infusion had a significant effect, but less than when it was given both prophylacticly and therapeuticly.

The cathepsin B content of the pancreas was slightly increased but not significantly by a supramaximal dose of caerulein, and E-3123 reduced this elevated level of cathepsin B somewhat, but not significantly (Table 1).

LDH and amylase discharge from dispersed pancreatic acini

A supramaximal dose of caerulein caused a significant increase in LDH discharge from dispersed acini in vitro, significantly higher at 60, 90, and 120 min than the control. The infusion of 2 or 5 mg/kg・hr of E-3123 before and during caerulein infusion, or only during caerulein infusion, significantly reduced this LDH discharge from dispersed acini, especially at longer incubation times (≧60 min). The administration of E-3123 at a dose of 1 mg/kg・hr had some protective effect, but it was not significant (Table 2). The amylase discharge from dispersed acini was also increased by a supramaximal dose of caerulein. The infusion of E-3123 in doses of 2 or 5 mg/kg・hr before and during caerulein infusion or 5 mg/kg・hr only during caerulein infusion significantly prevented the increased in amylase discharge from dispersed acini, especially at longer incubation times (≧60

Table 3 Effect of E-3123 on amylase discharge from dispersed acini during acute pancreatitis induced by supramaximal dose of caerulein.

Group	n	Amylase discharge from dispersed acini (% of total) Incubation time (min)			
		30	60	90	120
CER	6	10±1	23±2	32±3	41±4
E_1	6	10±1	19±2	27±2	34±3
E_2	6	8±1	16±2*	26±2*	31±2*
E_3	6	7±1	15±2*	23±2*	30±2*
E_4	6	9±1	17±2*	25±2*	32±2*
CONT	5	7±1	14±2**	22±2**	28±3**

The values are expressed as mean±SEM and the percent of total activity.
CER, caerulein group; E_1–E_3, caerulein plus E-3123 in doses of 1, 2 and 5 mg/kg・hr. E_4; caerulein plus 5 mg/kg・hr of E-3123 only during caerulein infusion; *, $p<0.05$; **, $p<0.02$, compared with CER group.

Table 4 Effect of E-3123 on lysosomal fragility induced by supramaximal dose of caerulein in the rat.

Group	n	Cathepsin B leakage from lysosomes (% of total) Incubation time (min)			
		30	60	90	120
CER	6	11±2	24±3	37±3	56±4
E_1	6	10±2	21±2	29±3*	45±3*
E_2	6	10±2	16±2*	26±2*	40±3**
E_3	6	8±2	15±2*	23±2**	37±3**
E_4	6	9±2	17±2*	25±2**	39±4**
CONT	5	7±2	13±2**	20±2**	34±3**

The values are expressed as mean±SEM and the percent of total activity.
CER, caerulein group; E_1–E_3, caerulein plus E-3123 in doses of 1, 2 and 5 mg/kg・hr; E_4, caerulein plus 5 mg/kg・hr of E-3123 only during caerulein infusion; *, $p<0.05$; **, $p<0.02$, compared with CER.

Table 5 Effect of E-3123 on mitochondrial fragility induced by supramaximal dose of caerulein in the rat.

Group	n	MDH leakage from mitochondrias (% of total) Incubation time (min)			
		30	60	90	120
CER	6	13±2	26±3	39±3	58±4
E_1	6	12±2	21±2	30±2*	44±3*
E_2	6	10±2	19±2*	27±2*	40±4**
E_3	6	9±2	17±2*	25±2**	38±3**
E_4	6	11±2	18±2*	28±3*	42±3**
CONT	5	9±2	16±2**	24±2**	36±4**

The values are expressed as mean±SEM and the present of total activity.
CER, caerulein group; E_1–E_3, caerulein plus E-3123 in doses of 1, 2 and 5 mg/kg·hr; E_4, caerulein plus 5 mg/kg·hr of E-3123 only during caerulein infusion; *, $p<0.05$; and **, $p<0.02$ compared with the CER group.

min). The administration of E-3123 in a dose of 1 mg/kg · hr had some protective effect, but it was not significant (Table 3). For the inhibition of cellular fragility induced by a supramaximal dose of caerulein at least 2–5 mg/kg · hr of E-3123 seems to be needed.

Cathepsin B leakage from lysosomes and MDH leakage from mitochondria

Supramaximal stimulation by caerulein also increased the cathepsin B leakage from lysosomes. The administration of E-3123 in a dose of 2 ot 5 mg/kg · hr before and during caerulein infusion, or 5 mg/kg · hr only during caerulein infusion prevented dose-dependently and significantly the cathepsin B leakage induced by a supramaximal dose of caerulein especially at longer incubation times (≧90 min) (Table 4).

A supramaximal dose of caerulein increased MDH leakage from mitochondria. The administration of E-3123 in a dose of 1, 2, or 5 mg/kg · hr before and during caerulein infusion, or 5 mg/kg · hr only during caerulein infusion prevented dose-dependently and significantly in MDH leakage from mitochondria induced by a supramaximal dose of caerulein, especially at longer incubation times (≧90 min). The combined prophylactic and therapeutic use of E-3123 in a dose of 5 mg/kg · hr provided the most protective (Table 5).

Discussion

There have been many other studies[4,6,20,25] both experimental and clinical on the crucial roles of proteases in the pathogenesis of acute pancreatitis. It seems, therefore, to be important to evaluate the protective effects of a new synthetic protease inhibitor on the exocrine pancreas in the early phase of the disease because the use of aprotinin and other inhibitors have had no effect on acute pancreatitis[11]

Furthermore, in acute pancreatitis, colocalization of lysosomal hydrolases and digestive enzymes in large cytoplasmic vacuoles has been reported to precede the appearance of cell injury[18,26]. Since the lysosomal hydrolase, cathepsin B, can activate trypsinogen[5,7,16], and trypsin can activate many other enzymes, this colocalization phenomenon might result in intracellular digestive enzyme activation and autodigestion of acinar cells.

E-3123 is a new synthetic guanidino acid ester, which inhibits a number of enzymes, such as trypsin, phospholipase A_2, kallikrein, palsmin, thrombin and C_{1R} and C_{1S} esterases[8].

The relatively low molecular weight of E-3123 (508 daltons) suggested that it might penerate into pancreatic acinar cells from the vasculature and encouraged studies to evaluate its protective effect against pancreatitis at both cellular and subcellular levels. Its potential use in the clinical treatment of acute pancreatitis is also of interest.

E-3123 was found to prevent the hyperamylasemia, pancreatic edema, and congestion of digestive enzymes in the acinar cells after infusion of a supramaximal dose of caerulein. In addition, in this caerulein induced pancreatitis, subcellular fractionation studies showed that E-3123 prevented the increased cellular, lysosomal and mitochondrial fragility of pancreatic acinar cells. These results indicate the protective effects of E-3123 on the subcellular organelles inside acinar cells as well as on the cellular membranes.

As a result of these in-vivo and in-vitro observations, we conclude that the increased cellular and lysosomal fragility are due to some as yet unknown protease activity, which can be inhibited by E-3123, and that this lysosomal and mitochondrial fragility is probably closely related to the pathogenesis of acute pancreatitis.

Recently some humoral factor which seems to play an important role in caerulein-induced experimental acute pancreatitis has been reported[19], and candidate proteases should include phospholipase as well as proteases that can be inhibited by E-3123. Further identification and characterization of that protease activity may shed important light on the pathophysiology of acute pancreatitis.

In conclusion, the favorable results shows in this study on cellular and lysosomal fragility in the early phase of secretagogue-induced experimental pancreatitis may justify an evaluation of E-3123 in a controlled clinical study.

Acknowledgments

This study was supported by a grant from the Ministry of Health and Welfare and a grant B-03454319 for Scientific Research from the Ministry of Education, Science and Culture of Japan. The authors thanK Ms. Kimiko Hirano for preparing the manuscript and Ms. Yoko Manabe for typing the manuscript.

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和文抄録

ラットセルレイン膵炎時の膵腺房細胞内小器官の脆弱性に対する新合成プロテアーゼ阻害剤 E-3123 の保護効果

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ラットセルレイン誘起実験膵炎の，in-vivo および in-vitro の両系にて，新しい合成 protease inhibitor である E-3123 の膵線房細胞への保護効果を検討した．E-3123 は，高アミラーゼ血症，膵浮腫，アミラーゼの膵内でのうっ滞を防止したのみならず，分離腺房細胞よりの LDH 漏出，ライソゾームよりの cathepsin B 漏出および，ミトコンドリアよりの MDH 漏出をも防止した．その効果はほぼ dose-dependent であったが，2 および 5 mg/kg・hr の容量にて特に著明であった．さらに，予防的＋治療的投与法が最も効果的であった．これらの結果は E-3123 の膵炎に対する保護効果がライソゾーム，ミトコンドリアや細胞膜および細胞内小器官の膜といった，細胞内レベルで発揮されていることを示唆させるとともに，臨床での急性膵炎の治療においてこれらの低分子量の合成 protease inhibition の有効性をも示唆させるものであった．